

Novel study of trace elements**Study of trace elements in BioArena system and
in in vivo conditions****Ernő Tyihák¹, Ágnes M. Móricz¹, Péter G. Ott¹, and Emil Mincsovcics²**

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Abstract: The adsorbent layer system is especially suitable for the biological evaluation of different compounds and trace elements as well. Present experiments showed that formaldehyde (HCHO) molecules participate in the antibiotic activity of Cu (II) ion, an „old antibiotic”. The elimination of HCHO from the chromatographic spots (e.g. by reduction or capturing) resulted in a characteristic decrease of the antibiotic effect of trace elements. The trace elements are HCHO carriers and generate a double effect (first step: deprivation of HCHO as also biological effect; second step: release of HCHO with big killing activity). These features offer good opportunities for influencing fundamental biochemical pathways. It has been established that the trace elements (mainly transition metal ions as e.g. Ni(II) ion) always generate quadruple, bioequivalent, specific immune-stimulating activity in plants with

a non-linear dose-response. HCHO and its reaction products (mainly O₃) are responsible also for this latter activity.

Key Words: antibiotic effect, BioArena, formaldehyde (HCHO), immunization, overpressured layer chromatography (OPLC), ozone, quadruple bioequivalent immune system, trace elements

INTRODUCTION

Trace elements (metal ions) play an important and unique role in all living organisms and without their catalytic presence in trace or ultratrace amounts in many essential co-factors many basic biochemical reactions would not take place. ^[1-3] It is known that „free” trace elements can become toxic to cells dose-dependently and they can cause damage to cellular components when their concentrations surpass certain optimal (natural) levels ^[4,5]. Trace elements generate diverse biological activities and they are involved in the generation and elimination of different diseases. Although it seems that each of them has its own mechanism of action, it is believed that these mechanisms actually share common factors. ^[4,5]

It is already known that the possibility of the enzymatic (biological) and/or spontaneous (chemical) methylation/hydroxymethylation of trace elements means a special and unique contact between the biological and the inorganic world. ^[6,7] It has to be pointed out that methylated/hydroxymethylated trace elements are potential formaldehyde (HCHO) precursors (generators) and HCHO formed from them can participate in different endogenous reactions/interactions. ^[8,9] Enzymatic transmethylation takes place through HCHO ^[10] and demethylation practically always involves formation of HCHO. ^[11] There is a primary HCHO cycle in biological systems (Figure 3A) ^[12,13] in which the formation of the S-methyl group of

L-methionine takes place through HCHO originating from natural HCHO generators and the formation of HCHO from SAM is linked to trans-methylation.

It was observed that the diverse beneficial effects of trans-resveratrol (TR) as a typical stilbene derivative e.g. in grapes (wines), can originate from its double effect.^[12,14] The elimination of uncontrolled and/or controlled HCHO molecules from labile bonds in a given tissue (e.g. heart or brain tissue) with exogenous TR molecules (first step) might have a chemopreventive effect. The products of reaction between the TR and endogenous HCHO can produce a killing/inhibiting effect e.g. against pathogens or cancer cells (second step).^[15,16]

It is known that some trace elements (e.g. As and Se) can be methylated enzymatically using S-adenosyl-L-methionine (SAM) as a „methyl donor”^[17] and spontaneously by means of HCHO formed/mobilized from cells/tissues. Trace elements (e.g. Cu and Zn) can be hydroxymethylated by means of HCHO originating from pathogen cells or from normal tissues etc. These methylation and hydroxymethylation reactions are analogous with the first step of the above TR reaction.^[15,16] The reaction products and further products (e.g. H₂O₃, O₃) can show the second step effect (see above) or they can participate in carcinogenesis (e.g. high dose of Cu coordinated with high HCHO level).

It follows from these observations that there are no determinative second step effects without interactions in the first step. The double effect may be the basis of diverse biological activities of trace elements.^[18] The killing/inhibiting effect of HCHO mobilized by trace elements can be further increased by means of interaction with H₂O₂. Both HCHO and H₂O₂ can be formed intracellularly and extracellularly by cells. These two small molecules can interact: singlet oxygen (¹O₂) and excited HCHO can form.^[19,20] ¹O₂ can oxidize water molecules and – among others- ozone can be formed which is an important component of the adaptive immune system (Figure 3B).^[21-23]

According to preliminary experiments and observations trace elements as carriers transport HCHO molecules in a dose-dependent manner to different points of a given biological unit. ^[24]

The known dramatic diversity of biological activities of trace (mainly transition) elements demands a deeper knowledge and understanding about their interactions with key biological small (and big) molecules. This paper illustrates in vitro (BioArena) and in vivo (greenhouse) systems that can be used to study these reactions.

EXPERIMENTAL

Experimental chemicals

Authentic test substances (e.g. trace elements, formaline solution) were purchased from REANAL Co., Ltd. (Budapest, Hungary). All solvents and other chemicals were of analytical grade, and purchased from Merck Co., Ltd. (Darmstadt, Germany) and Reanal Co., Ltd. (Budapest, Hungary). Dye reagent: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich Ltd., Budapest, Hungary) was used to visualize the antimicrobial activity on the adsorbent layer.

Layer Liquid Chromatographic Studies

TLC chromatoplates covered with silica gel 60 F₂₅₄ (Merck, Darmstadt, Germany) and sealed at all edges were used for OPLC separation. Sample application was carried out on preconditioned (3 h, 130 °C) adsorbent layers with a microsyringe and Linomat IV automatic sample applicator (CAMAG Co., Muttensz, Switzerland). TLC chromatograms are developed in an unsaturated chamber using different solvent systems. For OPLC separation, an automatic OPLC instrument was used, which consists of a liquid delivery system and a

separation chamber (OPLC-NIT Co., Ltd., Budapest, Hungary). A cassette containing the chromatoplate with samples can be inserted into the chamber. After the separation process using different solvent systems, the cassette is pulled out. The dried chromatoplates were ready for densitometric and/or bioautographic evaluations.^[25] In some cases the chromatoplates had to be impregnated with 0.3 M Na-molibdate solution for the moving trace elements from the start point.

Biological Detection of Trace Elements on the Adsorbent Layer: the In Vitro BioArena Studies

For the biotest, a bacterial cell suspension of the phytopathogenic *Pseudomonas savastanoi* pv. *phaseolicola* race 6, causing halo blight on bean, was applied. Just before use, different endogenous and/or exogenous key compounds, such as HCHO and O₃ capture molecules, were added to aliquots of the suspension. The developed, dried chromatoplates were immersed in the bacterial suspensions [with or without (control) endogenous or exogenous compound] for 25 s. Visualization of the bioautograms with MTT vital dye reagent was performed either after a short draining period or after an overnight incubation.^[26,27] After staining, the time for evaluation was varied from 1 h to a few days.

In Vivo Studies: Greenhouse Experiments - Biochemical Immunization of Plants

Chemical pre-treatment of different bean varieties was carried out by spraying the abaxial leaf surface of the plants with aqueous solutions of the inducer (+ 1 drop of TWEEN 40 in 100 mL solution) using decimal aqueous dilutions (ranged from 10⁻¹ to 10⁻²³ mol L⁻¹). Plants treated with water were used as a control. After an induction time period of 4 days bean plants were

spray-inoculated with an aqueous spore suspension of *Uromyces phaseoli* and then incubated (22 °C, 100 % relative humidity) for 24 h. ^[28,29] Evaluation of data was accomplished on the 9th day after inoculation. Rust pustules were counted by using a home-made pattern. Disease severity (infection rate, infectivity) was expressed as the average number of pustules on a 1 cm² leaf area (data on the statistical evaluation on a leaf surface of a minimum of 16 x 1 cm² are needed). These values are compared with the corresponding values of control plants. The obtained relative infection rate was expressed as a percentage (control 100 %). In order to study the effect of the inducer on the disease resistance of bean plants, the dependence of the infection rate on the concentration of the inducer administered (expressed in logarithmic scale) was examined ^[16, 28,30] The mathematical evaluation of the data was performed by moving average technique using suitable software.

(Of note, for successful experiments the chemical pre-treatment has to be carried out with an intensive washing of equipment between dilution steps with the actual dilution solution. This procedure includes a minimum of 10-15 times sprayings of next dilution solution into air or onto a indifferent surface.)

RESULTS AND DISCUSSION

Short theoretical introduction to the novel experiments

Figure 1 illustrates a modified Haraguchi ^[31] schematic model of the biological world where a biological cell and biological fluid are separated by the cell membrane. Our model version includes one mechanism of action for trace elements integrating HCHO and its special reaction products (e.g. ¹O₂, O₃) as well. This „chromatographic spot-like system” may be a home of most different biochemical pathway systems, at that time the layer chromatographic separation techniques (mainly in BioArena versions) can be used for studying them. Figure 1

shows that the metal ions (mainly transition metals) (M) [and parallel HCHO molecules (F) and their reaction products] which play a fundamental role in their mechanism of action weave through the big biological units such as the genome, proteome etc. with corresponding consequences. More recently, it has been observed that the well-known antimicrobial activity of Cu (II) ions is indirect: the Cu (II) ions generate (mobilize) HCHO molecules from microbial cells or plant tissues and bind them forming an unknown coordination complex. This also may be a biological action (HCHO deprivation) (first step in the interaction). ^[18] Figure 2 illustrates a structure of copper-hydroxymethyl complex which is a typical coordination complex of Cu (II) ion with a coordination number 6 forming a distorted octahedral geometry 1), while, the complex $[\text{Cu}(\text{CH}_2\text{OH})_4]^+$ has a tetrahedral geometry 2) taking into account excellent work of Theophanides and Anastassopoulou ^[4] with the copper-amine complex. In this coordination complex from the labile hydroxymethyl groups very reactive HCHO can be released (second step in the double effect) ^[18] with high killing/inhibiting activity to pathogen cells or to cancer cells. (It is supposed that other transition elements can generate similar hydroxymethylated derivatives in model reactions and in biological units, because all can mobilize HCHO molecules from microbes or other cells (tissues) in structure- and dose-dependent level. ^[18])

There is a primary HCHO cycle in biological systems (Figure 3a) ^[13,32] in which the formation of the S-methyl group of L-methionine takes place through HCHO originating from natural HCHO generators (e.g. N^5CH_3 (THF)) and the formation of HCHO from S-adenosyl-L-methionine (SAM) is linked to trans-methylation reactions ^[10] in general. HCHO cycle is a determining biochemical pathway in biological systems with unique, practically yet unknown functions. The inhibiting-killing activity of HCHO released can be further increased by means of interaction with an other endogenous very reactive molecule, H_2O_2 . Figure 3b summarizes

the reaction series supposedly taking into account the earlier and more recent determining observations.^[19-23] This complex figure includes two fundamental, related biochemical pathways which can be studied in BioArena system.

This study of trace elements includes the following new investigations: 1) basal reactions of trace elements with HCHO; 2) influencing activity of trace elements for the biochemical pathways; 3) study of trace elements in in vivo conditions based on BioArena studies.

Basal reactions of trace elements with HCHO and others in BioArena system

It has to be pointed out that column systems (e.g. HPLC columns) are not suitable for the biological detection of trace elements or organic compounds because the living cells (e.g. bacterial cells) do not grow there, so their detection and measurement (e.g. changes) is not possible. The BioArena system provides a solution here. It integrates the advantages of layer liquid chromatographic separation (ideally linear OPLC versions^(33,34)) and conventional and modern bioautography,^[26,35] which exploits the possibility to study interactions of cells (e.g. bacterial cells) with endogenous and exogenous small and large cofactor molecules in the adsorbent bed of the layer after separation. In these interactions HCHO and its reaction products (e.g. O₃) appear as key biological molecules.

Figure 4A illustrates the antibacterial activity of Cu (II) ions as a well-known „old antibiotic”. When HCHO-reducing molecules (L-ascorbic acid as a strong reducing agent) are added to the culture medium (Figures 4B, 4C, 4D) this activity is characteristically (non-linearly) reduced, in accordance with previous results.^[27] It seems that Cu (II) ions as old antibiotic molecules act through HCHO and its reaction products similar to organic antibiotic-like compounds.^[36]

Figure 5 shows that when HCHO molecules are added to the culture medium, the antibacterial activity is characteristically increased (more intensive spots are observable). This means at that time that the bacterial cells and culture medium do not provide enough HCHO molecules for the Cu (II) ions and shows that HCHO molecules participate in the antibiotic activity of Cu (II) ions (this is not a synergistic effect).

When natural („classical”) HCHO-capturing molecules (reduced glutathione and L-arginine) are added to the culture medium (Figure 6B and 6C) the antibacterial activity is decreased characteristically and especially considerably in the case of reduced glutathione in accordance with previous results of organic antibiotics. Cu (II) ions collect the HCHO molecules from the bacterial cells and the culture medium to reach an antibacterial (antibiotic) effect. ^[27,36]

Influence of trace elements on the biochemical pathways

Figure 7 shows the antibacterial activity of trans-resveratrol (TR) in the presence of Fe (III), Zn (II), and Mn (II) ions. These metal ions generated a bigger activity than the control in all cases, that is, they gave more HCHO molecules to effect of TR. The Cu (II) ions generated bigger effect than these trace elements. ^[37] It is obvious that the intensity of the first step (HCHO deprivation) depends on the trace element structure and dose but without this first step there is not a possibility for second step (killing/inhibiting effect). Importantly, trace elements generate double effect similar e.g. to TR. ^[15,16]

In the presence of mycotoxins (e.g. aflatoxins) the HCHO level in a given biological unit is elevated by advanced liberation from bounded forms (e.g. because of the stress situation) ^[13] and it may form from themselves aflatoxins by demethylation reaction. ^[29] The elimination of HCHO from the system (e.g. chromatographic spots) can decrease the antimicrobial-toxic

effect of mycotoxins. ^[38,39] Figure 8 illustrates the change of B1 aflatoxin antibacterial activity in the presence of different doses of Se (IV). It can be seen that increasing the amount of Se (IV) ion in the culture medium the antibacterial activity of aflatoxin B1 decreases dramatically. Meanwhile it has been established that Cu (II) and Se (IV) ions generate opposite effect on the antibacterial-toxic action of mycotoxins. ^[40]

It is known that trans-resveratrol (TR) is a natural, concentration—dependent HCHO mobilizer, scavenger, capture and carrier molecule. ^[15,16] It is interesting that in the presence of Cu (II) ions the antibacterial activity of TR considerably increases. ^[27,36] This is valid for other transition elements as it can be seen on Figure 7. Figure 9 illustrates the effect of cobalt (Co (II)) ions on the antibacterial effect of TR. It was probable that this transition element will also increase the antibacterial activity of TR, however, it seems that it acts perceptibly for the separation of two TR isomers as well. These results show clearly that the separation and observation possibilities are unlimited in BioArena system.

Ochratoxin A (OA) kills the pathogenic bacterial cells by endogenous ozone (O₃). ^[27,36,41] Figure 3B shows the formation possibilities of endogenous O₃ practically from the interaction of HCHO with H₂O₂. ^[19-23] Using Cu (II) ions in the culture medium the antibacterial activity of OA increases (Figure 10.) supposedly on the basis of reaction series demonstrated in Figure 3B. (HCHO is an O₃ precursor in this case).

Extension of in vitro (BioArena) results with trace elements for in vivo conditions (greenhouse investigations)

According to our preliminary investigations *in vitro* (BioArena – layer chromatography) results with trace elements can also be used in in vivo conditions [18,28,42] as in the case of organic compounds. ^[16,28,30] Figure 11 illustrates the quadruple, bioequivalent(four equal

immune-stimulating ranges), non-linear, specific (time- and dose dependent) immune response effect of bean plants for the pre-treatment with different doses of Ni (II) ion. These results support the preliminary results with the Cu (II) and Ni (II) ions as inorganic inducers.^[42] Using a HCHO capture molecule in vivo (e.g. dimedone), the four active immune response ranges of the bean plants could be eliminated,^[42] similar to the elimination of the antibiotic effect in the in vitro (BioArena) studies on the adsorbent layer.^[27] In fact the quadruple immune response of plants can analogously be induced by other trace elements. The possibilities are unlimited.

Figure 11 illustrates clearly that Ni(II) ions can also generate negative arm of hormesis effect ($-\log c = 1$) (retardation/destruction) and at that time the positive arm of the hormesis (immune-stimulating effect at $-\log c = 5$) as well.^[42]

CONCLUSIONS

The layer liquid system is suitable only for the biological detection and interactions, and development of BioArena provides unlimited possibilities for studying fundamental biochemical mechanisms. These possibilities ensure also the future of the layer liquid chromatographic techniques.

These results with trace elements (metal ions) challenge also the idea on the two-phase hormesis^[43,44] and show that hormesis phases are in the resistance phase of the stress syndrome.^[45] It seems on the basis of quadruple immune response of plants to pathogens that the resistance phase of stress syndrome^[45] can be divided into four equivalent parts.

Further study of trace elements (mainly transition metal ions) in vitro (in BioArena) and in vivo (e.g. in greenhouse) conditions regarding to HCHO/O₃ idea promises further

surprising results –among others - in the field of hormesis and resistance.

It follows also from these results that dosing will occupy more important role in our life in future than in earlier times. The quadruple, bioequivalent, non-linear, specific immune response system (also for trace elements) opens new horizons, and we are only at the beginning to understand this unique finding.

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Captions to figures of Tyihák E. et al.: JLC&RT, 2014.

Figure 1. A schematic model of the biological world in „a chromatographic spot” using a modified Haraguchi model [31] with special emphasis on the unique role of HCHO/O₃ idea in the mechanism of action supposed of trace elements (metal ions).

Figure 2. The potential structure of the copper-CH₂OH complex.

1) Octahedral geometry; 2) tetrahedral geometry [4,24].

Figure 3. Joining two fundamental biochemical pathways: formaldehyde cycle [12,13,32]

(A) and generation of key oxidants from the interaction of HCHO and H₂O₂ [27,28,36] (B).

Figure 4. Effect of L-ascorbic acid (AA) as reducing agent on the antibacterial effect of

Cu (II) ions against *Pseudomonas savastanoi* pv. *phaseolicola* (Psm).

A: layer dipped into Psm cell suspension, control; B-D: 10, 50 and 100 mg AA in 100 mL cell suspension; Chromatographic conditions: chromatoplate : silica gel 60 F₂₅₄ (Merck) impregnated by 0.3 M Na-molibdate, drying; then preconditioning at 130°C for 3 h. sample application; eluent: 1 M Na-formate (rechromatography); Detection (visualization) was performed with MTT (methyl thiazolyl tetrazolium chloride).

Figure 5. Effect of HCHO aqueous solution administration on the antibacterial activity of

Cu(II) ions against *Pseudomonas savastanoi* pv. *phaseolicola* (Psm).

Chromatographic conditions: silica gel 60 F₂₅₄ (Merck), preconditioning at 130° for 3 h. Mobile phase: 0.1 M Na-formate. Visualization was performed with MTT. The real control is the diluted formalin solution alone (third sheet). Spots were not observable there, but dramatic spots were observable in the copper and formaline solution on the second sheet.

Figure 6. Effect of natural HCHO capturers on the antibacterial activity of Cu (II) ions against *Pseudomonas savastanoi* pv. *phaseolicola* (Psm).

Chromatographic conditions: silica gel 60 F₂₅₄ (Merck); preconditioning at 130 °C for 3 h. mobile phase: 1 M formate; visualization with MTT.

Figure 7. Effect of different trace elements on the antibacterial activity of trans-resveratrol.

A: *Pseudomonas savastanoi* pv. *phaseolicola*, alone, control, Psm; B: + 1 mg/mL FeCl₃.6H₂O; C: + 1 mg/mL MnCl₂.4 H₂O; D: + 1 mg/mL ZnSO₄ . 7H₂O. Chromatographic conditions: chromatoplate: silica gel 60F₂₅₄ (Merck, preconditioning at 120 °C for 3 h); mobile phase: chloroform-methanol, 80:8, (v/v); Incubation time between inoculation and staining was 2 h. This picture was taken 18 h after staining.

Figure 8. Effect of Se(IV) ions on the antibacterial activity of aflatoxin B1.

A: *Pseudomonas savastanoi* pv. *phaseolicola*, alone, control;

B-E: + 0.01, 0.1, 1 and 2 mg sodium selenite in 100 mL cell suspension. Chromatographic conditions: chromatoplate: silica gel 60F₂₅₄ (Merck, preconditioning at 120 °C for 3h; mobile phase: chloroform-acetone, 9:1 (v/v); Detection at the end of the process with MTT.

Figure 9. Effect of cobalt (II) ions on the antibacterial activity of trans-resveratrol.

A: Psm, *Pseudomonas savastanoi* pv. *phaseolicola* cell suspension, alone, control; B-D: + 2, 4 and 6 mg CoCl_3 in 100mL cell suspension

Chromatographic conditions: silica gel 60 F₂₅₄ (Merck). Preconditioning at 130 °C for 3 h. Mobile phase: chloroform-methanol 80:8 (v/v) Visualization with MTT.

Figure 10. Effect of Cu(II) ions on the antibacterial activity of ochratoxin A.

A: Psm, *Pseudomonas savastanoi* pv. *phaseolicola* cell suspension, alone, control; B and C: 4 and 6 mg $\text{CuSO}_4 \times 5\text{H}_2\text{O}$ in 100 mL cell suspension

Chromatographic conditions: silica gel 60F₂₅₄ (Merck). Preconditioning at 130°C for 3 h. Mobile phase: chloroform-methanol, 8:2 (v/v). Visualization with MTT.

(Copper ion was the HCHO carrier and OA is a HCHO acceptor molecule on the basis of its structure.)

Figure 11. Effect of Ni (II) ion as inorganic inducer on the disease resistance of

bean plants to bean rust (*Uromyces phaseoli*) using decimal dilution.

Bean plants are inoculated with an aqueous spore suspension of bean rust 4 days after pre-treatment with the doses of inorganic inducer. (Ni (II) ion is a HCHO carrier!)

(This figure is a demonstration of quadruple, bioequivalent, non-linear, specific immune response system of plants). (Induced resistance)

Figures to JLC &RT, Tyihák E. et al. (2014)

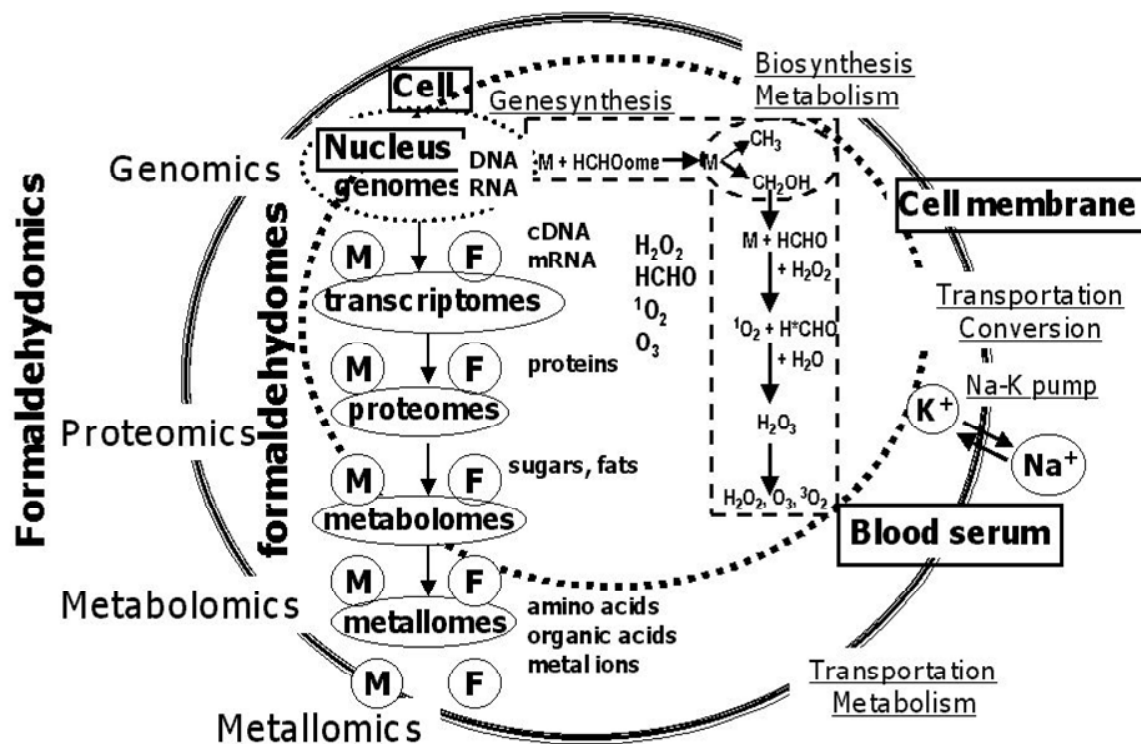


Figure 1

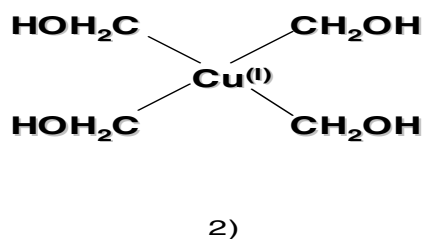
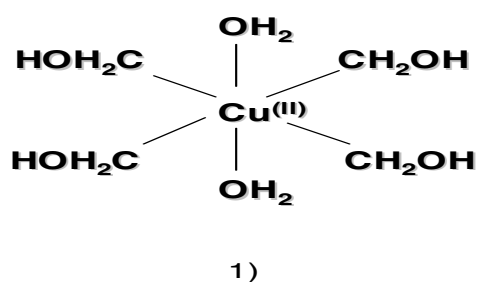
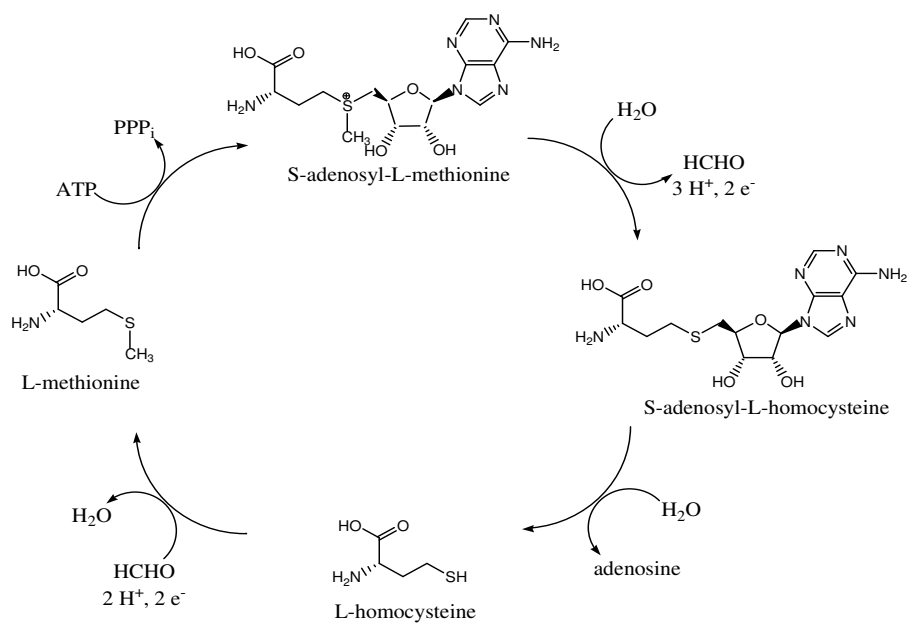
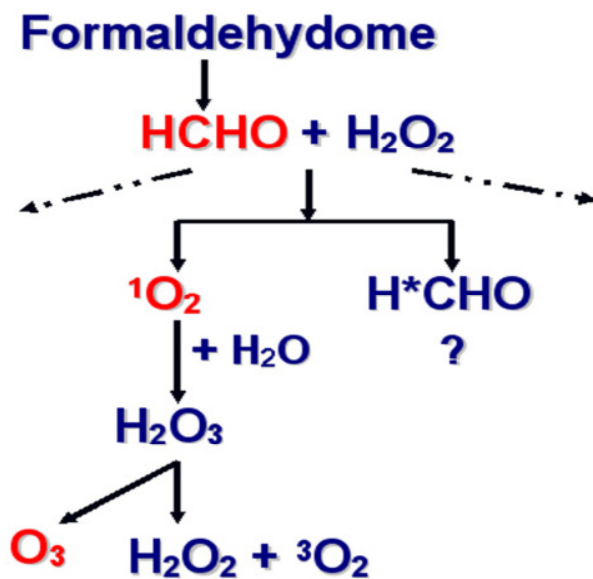


Figure 2



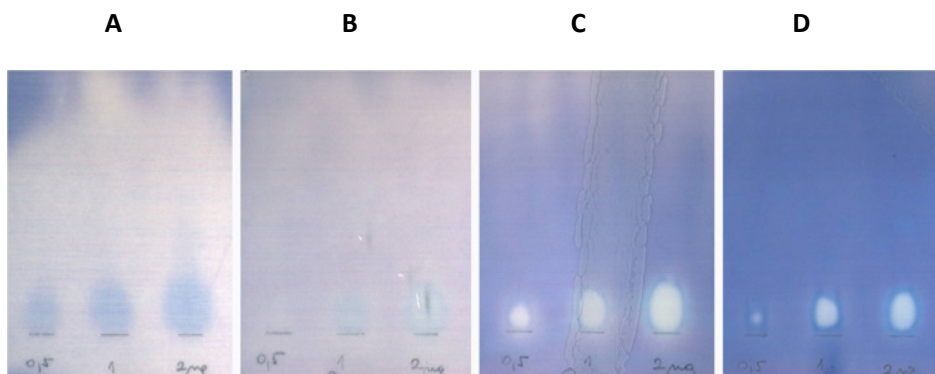
A) HCHO cycle

through the formaldehyde system



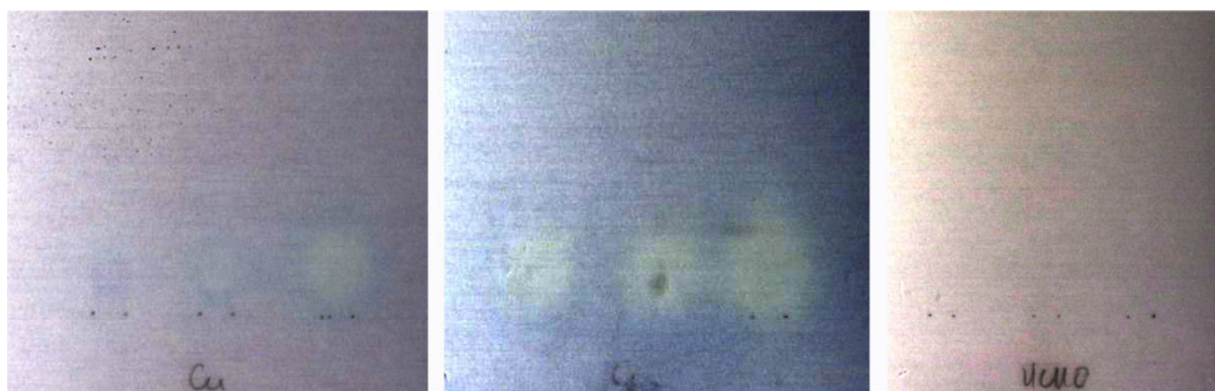
B) Simplified reaction scheme of reactive oxidants

Figure 3



0.5, 1.0 and 2.0 µg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (in all four cases)

Figure 4



1, 2 and 4 µL of 1 mg/mL

$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$

dissolved in distilled water

1, 2 and 4 µL of 1 mg/mL

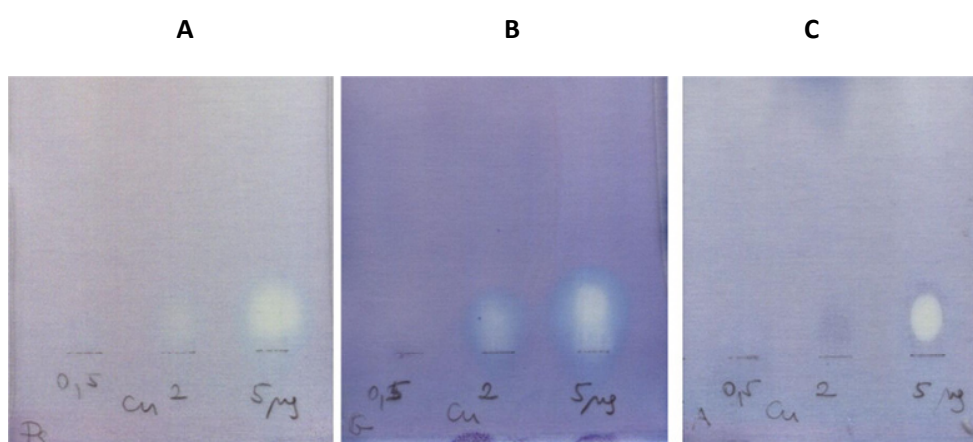
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$

dissolved in 0.316 M formalin

1, 2 and 4 µL of

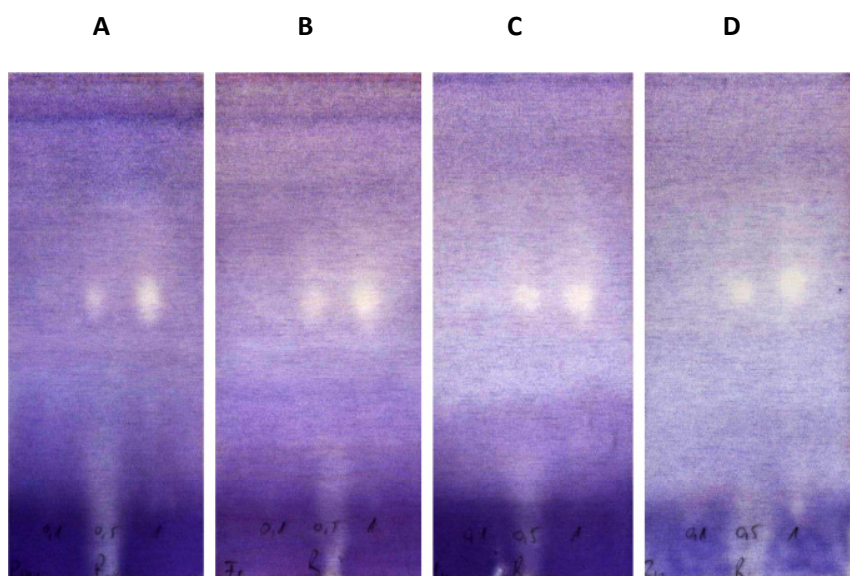
0.316 M formalin

Figure 5



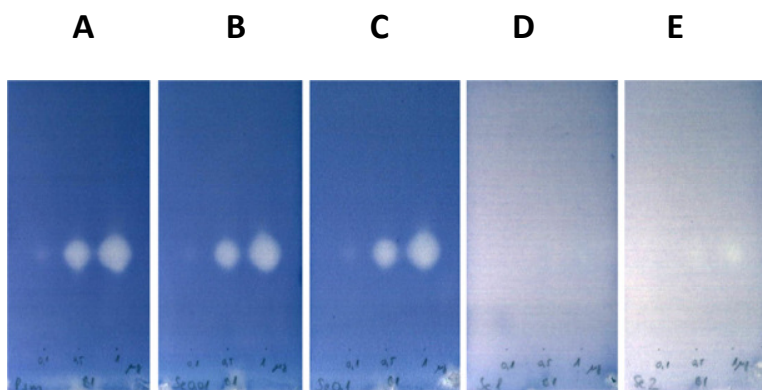
0.5, 2.0 and 5 μg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (in all three cases)

Figure 6



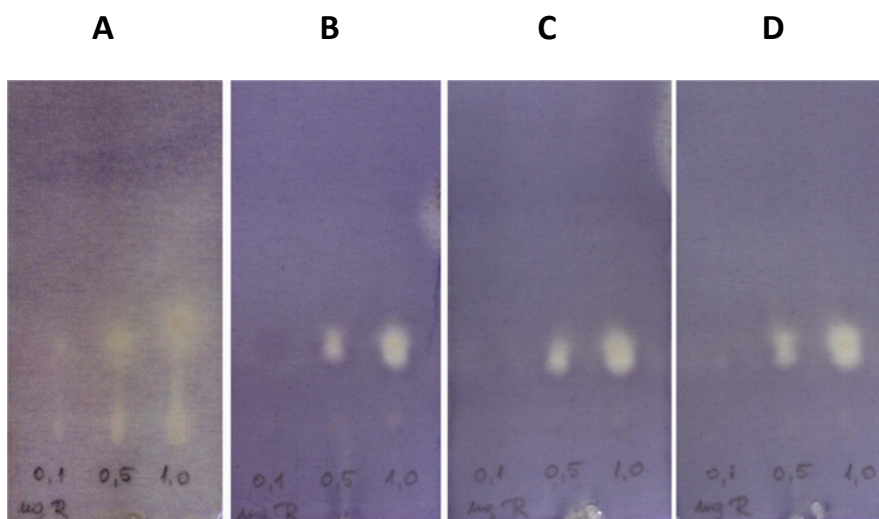
0.1, 0.5 and 1 μg trans-resveratrol (in all four cases)

Figure 7



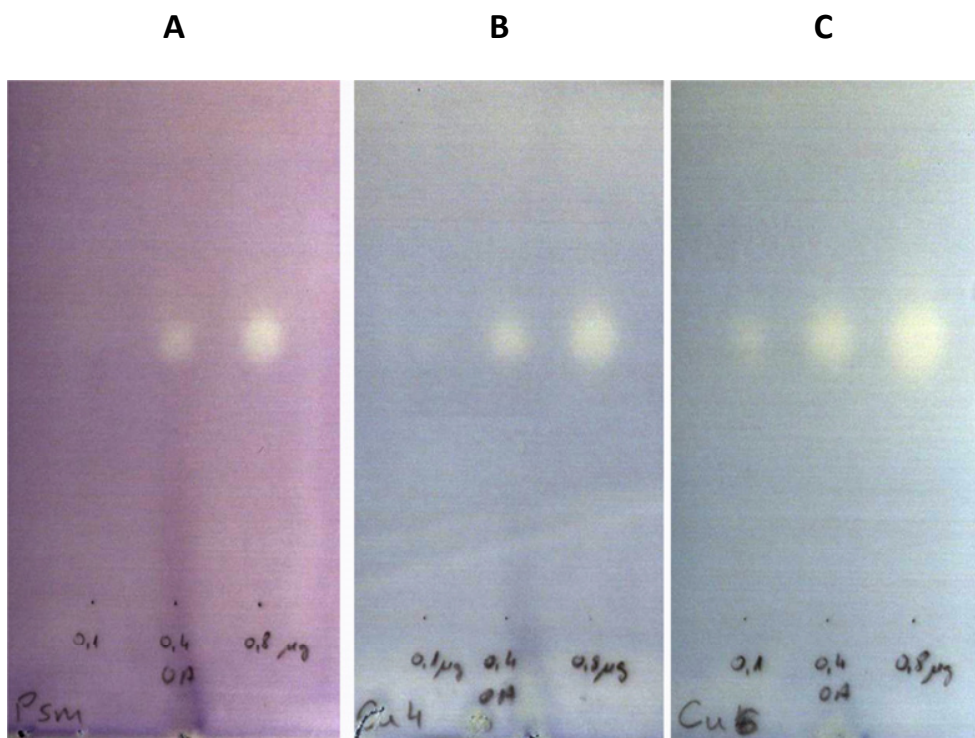
0.1, 0.5 and 1.0 μg aflatoxin B1 (in all five cases)

Figure 8



0.1, 0.5 and 1.0 μg trans-resveratrol (in all four cases)

Figure 9



0.1, 0.2 and 0.8 µg ochratoxin A (in all three cases)

Figure 10

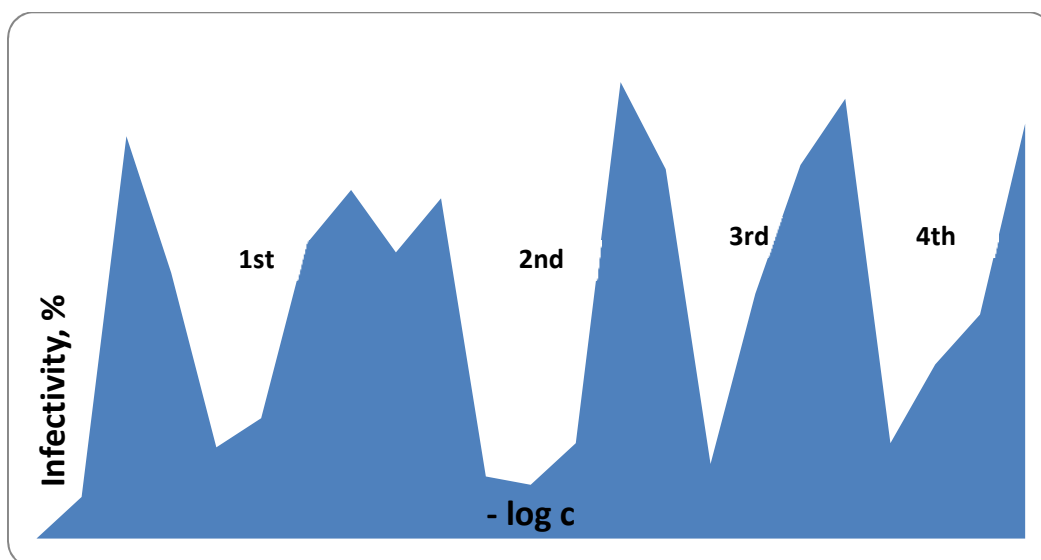


Figure 11